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(71)(72) Applicants and Inventors: WOLFF, Jon. A. [US/US]; 1122 University Bay Drive, Madison, WI 53705 (US). JIAO, Shou, Shu [CN/US]; 27 University Houses, Apt. D, Madison, WI 53705 (US).

(74) Agent: SEAY, Nicholas, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).

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(54) Title: METHOD OF DELIVERING THERAPEUTIC SUBSTANCES TO THE BRAIN

(57) Abstract

A method for delivering therapeutic substances to a patient's brain is disclosed. This method begins with isolating a nucleotide sequence, wherein said nucleotide sequencing encodes a protein of therapeutic value. Muscle tissue is isolated and transformed with the nucleotide sequence. The transformed muscle tissue is then implanted into the brain of a patient, whereupon said nucleotide sequence is expressed and a therapeutic substance is produced by the transformed cells.

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METHOD OF DELIVERING THERAPEUTIC SUBSTANCES TO THE BRAIN

Field Of The Invention

The present invention relates to treating disease via gene therapy. Specifically, the present invention relates to the treatment of a patient's disease through the implantation of transgenic muscle cells engineered to deliver therapeutic substances into a patient's brain.

Background

Intracerebral transplantation of genetically-modified tissue has been proposed for the treatment of several genetic and acquired neurologic disorders. Gage _et al_., _Neurosci_., 23:795-807 (1987) and U.S. Patent No. 5,082,670. These disorders include Parkinson's disease and Alzheimer's disease. The genetically-modified cells would not necessarily alleviate the neurologic symptoms by establishing synaptic, point to point connections, but would function instead by secreting therapeutic proteins, such as NGF, or metabolites, such as L-DOPA into the cerebral fluids. In addition, it may not be required that these proteins or metabolites be secreted in a regulated fashion, but instead can be expressed by unregulated, constitutive pathways that are common to all cells. Thus, many types of cells have been proposed as an intracerebral "platform" for the production of therapeutic substances.

Neuronal, glial, skin, and endocrine tissue has been transplanted into rat brain for the study of neurophysiology and for the development of new therapeutic

modalities. Gage, et al., Neuron, 6:1-12 (1991); Gash, et al., Neurobiology of Aging, 6:131-150 (1985). A critical factor in the usefulness of these transplants is the longevity of the transplanted tissue. Long-term survival has been documented for fetal neuronal (Bakay, et al., Stereotact. Funct. Neurosurg. 53:1-20 (1989)) and adrenal cells (Freed et al., J. Neurosurg., 65:664-670 (1986)). Studies involving skin or glial cells are in progress (Chen et al., J. Cell. Biochem., 45:252-257 (1991), LaGamma et al., Soc. Neurosci. Abstr., 17:572 (1991). Gage et al. (U.S. patent 5,082,670) discloses a method of grafting transgenic cells to treat diseases of the central nervous system. Gage et al. (column 12, line 15-50) disclose the usefulness of many different cell types as choices for grafting, but do not list "muscle cells" as a choice.

Intracranial muscle grafts have been proposed for various therapies, but the muscle grafts did not survive long-term. Heinicke, Acta Neuropathol. (Berl), 49:177-185 (1980); Wakai, et al. Brain Res., 386:209-222 (1986). According to Wakai, et al., only those muscle autografts placed on the surface of the brainstem survived for at least one year. In the parenchymal muscle grafts (intrabrain muscle grafts), less than 10% of the grafts' volume was occupied by striated muscle cells and even this modest number of muscle fibers diminished with time. Copious, redundant basal, laminae, together with degenerated muscle cells, were common in the 1 and 3 month old parechymal grafts and the 12 month old grafts contained only degenerated muscle cells and connective tissue.

The transplantation of myoblasts into muscle has previously been proposed for the treatment of myopathies (Partridge, et al. Muscle & Nerve, 14:197-212 (1991)) and metabolic disorders (Smith et al., Mol. Cell. Biol., 10:3268-3271 (1990)). Transplanted myoblasts can persist long-term in animal muscle without spontaneous tumorogenesis. Therefore, clinical trials are now exploring the safety and efficacy of myoblast

transplantation in humans (Huard et al., Clin. Sci., 81:287-288 (1991); Law et al. Adv. Exp. Med. Biol., 280:75-87 (1990)).

A variety of methods have been used to genetically modify muscle cells both in vitro and in vivo by the insertion of a foreign gene (a "transgene") into such cells. The transgene can be expressed in a long-term manner in these cells (Smith et al., Mol. Cell. Biol., 10:3268-3271 (1990); Wolff et al. Science, 247:1465-1468 (1990); Yang et al. Proc. Natl. Acad. Sci. USA, 87:9568-9572 (1990)). These manipulations are supported by a relatively good understanding of the role and behavior of myoblasts and satellite cells during normal muscle development or during muscle pathology (Emerson et al., Molecular Biology of Muscle Development, Alan Liss, New York, 1986; Schultz, Med. Sci. Sports. Exer., 21:S181-S186 (1989).

What is needed in the art of disease treatment is a method of transplanting transgenic tissue into brain that provides long-term expression of a therapeutic substance.

Summary of the Invention

The present invention is a method for delivering therapeutic substances to a patient's brain. The method comprises the steps of first isolating a nucleotide sequence, wherein that nucleotide sequence encodes a protein of therapeutic value. Muscle tissues are then isolated and transformed with the nucleotide sequence. These transformed muscle cells are implanted into the brain of a patient, whereupon said nucleotide sequence is expressed.

In a particularly advantageous embodiment of the present invention, the transformation of the muscle tissue is by bombarding the muscle tissues with carrier particles coated with the nucleotide sequence.

The object of the present invention is to deliver therapeutic molecules to a patient's brain.

An advantage of the present invention is that a "platform" is provided for the long-term production of therapeutic molecules.

Other objects, advantages and features of the present invention will become apparent from the following specification when taken in conjunction with the accompanying drawings.

Brief Description of the Drawings

Fig. 1 is an exploded perspective view of an apparatus suitable for use in the present invention.

Fig. 2 is a top plan view of the apparatus of Fig. 1.

Brief Description of the Preferred Embodiments

The present invention relates to the use of transgenic muscle cells (myoblasts and myotubes) and muscle fiber as a "platform" for intracerebral transgene expression and the delivery of therapeutic substances to the brain by "therapeutic substance," we mean any therapeutic molecule, such as a protein or a secondary metabolite of protein activity, that will alleviate a disease state. This invention is of particular use to a patient who has a disease that can be treated by the delivery of therapeutic substances to brain cells. In brief, muscle tissue is excised from a patient. This tissue is prepared for transformation and transformed with a nucleotide sequence encoding a protein of therapeutic value. By "protein of therapeutic value" we mean that either the protein itself alleviates a disease state or produces a metabolite that alleviates a disease state. The transgenic muscle tissue is implanted in the patient's brain in a region of the brain in which the therapeutic protein is desired.

1. Preparation of Transgenic Muscle Tissue

The examples below describe two methods of preparing suitable muscle cells. These two methods involve excising muscle tissue from the patient and either mincing the muscle before implanting or enzymatically harvesting cells

from muscle tissue. A suitable source of muscle is the patient's quadriceps muscle because a small piece of muscle may be excised without harm to the patient, but other sources could be used.

For the minced muscle procedure, a small piece of quadriceps muscle is removed from the patient and placed into an appropriate buffer. This muscle is minced with a sharp instrument, such as sharp-pointed scissors, into small specimens. Preferably, these specimens are approximately 0.1 mm in diameter.

Enzymatic harvesting of muscles is preferably accomplished as in Yablonka-Reuveni et al. Dev. Biol., 119:252-259 (1987). Young adult or baby rats (3-5 days old) are anesthetized with 2.0 ml/100 gm body weight of 3.5% chloral hydrate (I.P.). The soleus muscle is removed using sterile technique and placed in a 60 mm sterile petri dish with a small volume of CMF Hank's solution (1X, Gibco). Muscle is rinsed with Hank's solution to remove any blood and then minced finely with sterile scissors. The minced muscle is then transferred to a 50 ml flask containing the muscle enzyme solution (0.169% trypsin, 0.085% collagenase, CMF Hanks, pH 7.4) that has been pre-warmed for 15 min at 37 °C and incubated for 55 min (for baby rat muscle, incubation time could be 20 min) while the flask is shaken every 15 min and the pH checked. Sterile 0.1 M NaOH is used to regain proper pH color when necessary. Contents of the flask are transferred to a plastic sterile test tube and centrifuged for 5 min at "4" setting on the clinical centrifuge. After 2 washings with complete media, NRM (normal rat media, 5% chick embryo extract <Gibco, BRL>, 15% horse serum, 80% modified Eagle's media), the pellets are resuspended in 2.5 ml NRM and mechanically dissociated through an 18-gauge needle. The resulting cell suspension is filtered through a nitex filter to eliminate myotubes and connective tissue, and then purified by Percoll (Sigma) gradient centrifugation. The muscle cell fraction is manually collected, diluted with NRM, and centrifuged in the clinical centrifuge for 10 min. After the resulting pellet is resuspended in an

appropriate volume of NRM, and the cell number is counted, the cells are plated out into 35 mm culture dishes (10^6 cells per dish). The cells are then maintained in an incubator (37 °C, humidified atmosphere of 5% CO_2 in air) with NRM media that is changed every other day.

The invention is directed toward the introduction of exogenous, typically chimeric, genetic constructions into muscle cells. Such exogenous genetic constructions consist of DNA from another organism, whether of the same or different species. The exogenous DNA construction would normally include a coding sequence for a transcription product or a protein of interest, together with flanking regulatory sequences effective to cause the expression of the protein in the transformed cells of an organism. Examples of flanking regulatory sequences are a promoter sequence sufficient to initiate transcription and a terminator sequence sufficient to terminate the gene product, whether by termination of transcription or translation. Suitable transcriptional or translational enhancers can be included in the exogenous gene construct to further assist the efficiency of the overall transformation process and expression of the protein result in the transformed muscle cells. A signal sequence, effective to cause secretion of the therapeutic protein into the intracellular space, might usefully be linked to the protein. The inserted construction could itself be RNA, as an alternative to DNA, if only transient expression of the gene product is desired.

In many applications, it is most advantageous if secretion of the therapeutic protein is achieved in transgenic cells. This can be accomplished by attaching to the 5′ end of the coding region for the protein of interest a signal peptide coding sequence. The signal peptide should preferably condition extra-cellular transport and cleavage so as to deliver the protein into the intracranial space.

The excised and treated muscle cells are then transformed with the exogenous nucleic acid construction. The art of mammalian cell transformation contains many

suitable methods. The examples below describe muscle cells transformed by injection with a DNA-containing solution, by a Lipofectin-mediated method and the transformation of muscle cells via bombardment with DNA-coated particles.

A suitable transformation method would produce enough transformed cells so that detection of the transformants is reasonably efficient. A suitable method would not damage the muscle platform cells to the extent that at least a portion of the cells could not be implanted and produce the desired protein.

Because the transformation event may not be highly efficient, it may be necessary to select or screen for muscle cells have been transformed. This objective may be accomplished with the use of suitable marker or selectable agent genes transformed into the platform muscle cells. These genes may be linked to the gene of therapeutic interest or may simply be co-transformed.

A marker gene encodes a gene product which can be easily assayed, such as beta-galactosidase. The presence of the product of the marker gene indicates that the cell is transformed. Even if the assay destroys the examined cells, information as to the percentage of transformants would be gained.

A selectable marker gene encodes a product which the cell would need to survive in a specific environment. An example of a selection gene would be a gene encoding antibiotic resistance. Therefore, the cells in question could be subjected to the antibiotic and only the transformed cells would survive.

2. <u>Preparation of Patients</u>

Once transformed muscle tissue has been obtained, the tissue must be implanted in the patient's brain. Preferably, a patient's own muscle will be used for brain implantation to avoid immune rejection, i.e. the transplant is autologous. Surgical techniques that are known in the art will be used to create a skull window in the section of the patient's brain into which the

transgenic implant is desired and the transgenic tissue will be implanted.

EXAMPLES

1. "Minced" Muscle Implants.

A. Preparation of Muscle

A 5 X 5 X 4 mm piece of a quadriceps muscle was removed from one- to two-month-old Lewis or Sprague Dawley rats and placed into phosphate buffered saline (PBS) containing 0.6% glucose at room temperature. Blood was removed by washing with this solution. The muscle was minced with a sharp-pointed scissors (Fisher Scientific) into specimens of 0.1 mm in diameter.

B. Preparation of Animals

The same animal served as a host for brain implantation of its minced muscle to avoid immune rejection. A 2.5 mm diameter skull window was opened in the rat using a dental drill. The window was 2 mm lateral to the bregma over the frontal lobe. The dura was carefully opened and a piece of the brain tissue was aspirated to make a cavity that was 2 mm in diameter and 2 to 3 mm deep. After application of gel foam (The Upjohn Co.) to sop the bleeding, the minced muscle was implanted with an 18 gauge metal cannula. The overlying skin was closed with subcutaneous sutures and clips.

C. Analysis of Implants

Four to six animals with minced muscle implants were sacrificed at one week, one month, two months, four months and six months to determine if the intrabrain muscle graft could survive long-term. Hematoxylin and eosin (H&E) staining of the brain sections revealed that muscle grafts were present at each of these time points.

From examining the stained grafts, we determined that the one-week-old muscle grafts were comprised of myotubes or small myofibers in approximately equal proportion. Few necrotic fibers were evident at this time. The two-

week-old grafts were mostly comprised of myofibers with a few myotubes at the periphery of the grafts. The grafts older than two weeks were comprised mostly of myofibers with striations. Thus, the transplanted cells were developing in a normal manner.

The progression from myotubes to myofibers in the brain grafts were similar to the progression observed when minced muscle was grafted into muscle (Grounds et al. J. Pathology, 132:325-341 (1980); Partridge et al. J. Neurol. Sci., 33:425-435 (1977)). However, the rate of the progression to myotubes and myofibers now observed in the adult muscle to brain grafts appeared faster than what was previously observed in adult muscle to muscle grafts. This rapid progression was more like that observed when neonatal muscle was grafted into muscle (Grounds et al., supra).

Fewer than ten percent of the myofibers in the grafts older than two weeks contained central nuclei. The rest of the myofibers contained peripheral nuclei. The large percentage of peripheral nucleation found in the myofibers is an indication of well-differentiated muscle cells. This peripheral nucleation was an unexpected finding because previous studies had indicated that grafts of minced, adult muscle grafted back into muscle contained an increased percentage of central nuclei (Grounds et al. supra). In fact, adult rodent muscle that has undergone regeneration under a variety of conditions typically contained myofibers with central nuclei. In contrast, minced neonatal muscle or neonatal myoblasts formed myofibers with peripheral nuclei when transplanted into muscle (Grounds et al., supra). Both the preponderance of myotubes at one week and the peripheral nucleation in the adult muscle to brain grafts were similar to the behavior of neonatal muscle cells. These observations suggest that possibility that the host brain may have provided factors that enabled the transplanted adult muscle to form myotubes more quickly and peripheral nucleated myofibers.

Brains that were similarly implanted with slivered but not minced muscle contained dying myofibers at one

week and a few myotubes at two weeks. Mincing may enable better vascularization of the grafts by disrupting the epimysium and perimysium.

All of the four brains for each time period contained substantial grafts. Measurement of the graft maximum diameter provided a semi-quantitative assessment of their size. The mean maximum diameters (± indicates standard error) of the grafts did not change over time and were 1.94 (± 0.15) mm at one week (n=4), 1.78 (± 0.11) mm at two weeks (n=6), 2.17 (± 0.22) mm at one month (n=5), 1.77 (± 0.10) mm at two months (n=6), 2.33 (± 0.34) mm at four months (n=4), and 2.13 (± 0.21) mm at six months (n=4) after transplantation. These results indicate that the muscle grafts were present in the brains for at least six months. All of the experimental animals survived without any obvious adverse effect.

The grafted, minced muscles were further studied by immunohistochemically staining of the brains for muscle myosin using MF20 anti-myosin antibody (Developmental Studies Hybridoma Bank, Baltimore, MD) and a FITC-conjugated, anti-mouse, IgG antibody (Sigma) as previously reported in Bader, et al. J. Cell Biol., 95:763-770 (1982). Only brains implanted with minced muscles contained cells with myosin staining in a striated pattern. Brains transplanted with unminced muscles had much less myosin staining.

We examined the grafts via electron microscopy. This technique showed that the muscle grafts contained normal myofilaments six months after transplantation.

In summary, the presence of striations and peripheral nuclei in six-month-old muscle grafts suggested that minced muscles survived long-term in a healt[illegible] and differentiated state.

D. Implantation of Transgenic Muscle [illegible]

We transplanted muscle cells that had been genetically modified by the intramuscular injection of plasmid DNA. Previous studies had shown that muscle cells

in vivo can take up and stably express plasmid DNA delivered to the extracellular space.

Quadriceps muscles were injected intramuscularly with 200 μg of the luciferase expression plasmid, pRSVL (De Wet et al., Proc. Natl. Acad. Sci. USA, 82:7870-7873 (1985)) as previously reported in Acsadi et al., New Biologist 3:71-81 (1991) and Wolff, et al. Science, 247:1465-1468 (1990). Four days after injection, the muscles were either analyzed directly for luciferase activity or implanted into the brain. Extracts of the quadriceps muscles or the frontal part of the recipient brains were prepared in 200 μl of lysis buffer and 20 μl of the supernatant was assayed for luciferase as previously reported in Acsadi et al. New Biologist, 3:71-81 (1991). The muscle extracts prior to transplantation contained a mean of 1,086,460 (± 206,729) Light Units ("L.U.") (n=5). At one day after transplantation, the recipient brains contained 70,697 (± 68,591) L.U. (n=4). The recipient brains contained only background levels of luciferase (<400 L.U., n=12) at seven days post-transplantation.

2. Enzymatically Harvested Muscle Implants.

A. Preparation of Myoblasts

Myoblasts were enzymatically harvested from one to three-day-old Lewis rats and purified by Percoll (Sigma) gradient centrifugation as in Yablonka-Reuveni et al., Dev. Biol., 119:252-259 (1987) as described above. The confluent myotubes in a 35-mm plate were harvested without trypsinization using a rubber policeman.

B. Implantation of Cells.

The muscle tissue was either implanted into the cortex or injected stereotaxically into the caudate nucleus of one- to two-month-old Lewis rats.

Adult male Lewis rats (200-300 gm) were used as recipient animals. The rats were anesthetized with intramuscular injection of a mixture of Ketamine (110 mg/kg) and placed securely in the stereotaxic instrument.

Intracaudate implantation consisted of the following: Myoblast or myotube suspensions were stereotaxically injected into the striatum of host rat brain at the following coordinates (in mm) relative to the bregma and the dural surface, and with the tooth-bar set at zero: A=0.2-0.7, L=2.5-3.0, V=5.5-6.5 (Paxinos and Watson, The Rat Brain in Stereotaxic Coordinates, 1982) The cell suspension was centrifuged and the pellet was resuspended in a volume of Basic Medium to yield approximately 40,000 vital cells per microliter. A total of 10 µl of the cell suspension (equivalent to a total of approximately 50 X 10^4 viable cells) was delivered to the striatum by a 50 µl Hamilton syringe with a 23 gauge needle over a period of 5 min. After each injection to the striatum, the needle was left in place for five min and slowly withdrawn from the brain. A small piece of gelfoam was inserted into the opening in the skull, and the scalp sutured.

Measurement of the maximum diameter of the graft provided a semi-quantitative assessment of the size of the graft over time. In the implanted group, the mean maximum diameters (± indicates standard error) of the grafts were 1.36 (± 0.08) mm at one week (n=6), 1.60 (± 0.11) mm at two weeks (n=6), 1.33 (± 0.20) mm at one month (n=6), and 1.28 (± 0.19) mm at six months (n=6) after transplantation.

Myosin staining (Bader, et al., J. Cell. Biol., 95:763-770 (1982)) and H&E staining of the grafts six months post-implantation confirmed that the muscle cells contained myosin and myofilaments. Brains injected with myotube cultures into the caudate nucleus also contained myosin-positive muscle cells after six months.

Electron microscopy revealed intact, nucleated muscle cells with myofilaments.

C. Implanting Transgenic Muscle Cells.

A method of transfecting myotubes in culture via liposome-mediated transformation was developed. Myotubes in a 35-mm plate, differentiated for 5 to 8 days, were transfected using Lipofectin as in Felgner et al., Proc

Natl. Acad. Sci. USA, 84:7413-7417 (1987). The myotube cultures were washed three times with serum-free Opti-MEM (BRL). After exposing the cultures to pre-mixed complexes of 15 μg of pRSVL and 45 μg of Lipofectin in 1.0-ml of Opti-MEM for four hours, 1.5-ml of complete medium was added. Three days post-transfection, the cultures contained 11,370,000 (± 1,598,376) L.U. of luciferase activity (n=6) which is approximately five to ten times greater than the activity obtained with 3T3 mouse fibroblasts transfected using similar methods. The myotubes were similarly transfected with the *E. coli* beta-galactosidase plasmid, pRSVLac-Z (Norton *et al.* Mol. Cell. Biol., 5:281-290 (1985)) to determine the percent of myotubes transfected. Approximately 50% of the myotubes transfected with 15 μg of pRSVLac-Z and 45 μg of Lipofectin were stained blue in this assay, thus indicating beta-galactosidase expression.

The transfected myotubes were implanted into adult Lewis rat brains. The sections of brains grafted with myotubes transfected with pRSVLac-Z were stained histochemically for beta-galactosidase activity as described in Acsadi, *et al.* New Biologist, 3:71-81 (1991). The muscle grafts contained beta-galactosidase-positive myofibers two weeks post-transplantation. More quantitative information was obtained using pRSVL-transfected myotubes. After implantation of pRSVL-transfected myotubes, mean luciferase activities (± standard errors, n=6 for each time period) were 167,142 (± 71,626) L.U. at seven days post-transplantation, 195,013 (± 70,697) L.U. at two weeks post transplantation, 150,537 L.U. (± 27,500) at four weeks, and 191,852 L.U. (± 33,233) at eight weeks post-transplantation. The contralateral side of the brain that did not receive implants contained background levels of luciferase (<400 L.U.).

Although luciferase expression decreased substantially from the pre-implantation levels of 11,370,000 L.U. to 167,142 L.U. seven days after transplantation, expression was stable from seven days through two months post-transplantation. The reason for

the large drop in luciferase expression soon after transplantation is probably due to damage to the myotubes during the transplantation process.

In summary, we established that grafted muscle cells survived in the brain for at least six months and that plasmid expression was stable for at least two months. The robust and consistent long-term survival of the transplanted muscle cells suggests that this cell type may be a useful "platform" for transgene expression in brains. Our experiments indicate that differentiated myoblast cultures transfected with plasmids can stably express a transgene in the brain for at least two months.

3. <u>Particle-mediated Transformation</u>

In a particle-mediated transformation method, cells or tissue are bombarded by nucleic acid-coated particles. The coated particles deliver foreign genetic material to the interior of the cell. The particle-mediated transformation procedure described in published PCT application WO 91/00359 is especially suitable for creating transgenic mammalian cells. This technique could be used to create transgenic muscle cells which could then be implanted in brain, as described above. Suitable muscle cells could be obtained as described above. The particle bombardment could occur either before or after the mincing or enzymatic digestion used to isolate the muscle tissue.

There are other particle-mediated transformation methods that could be equally suitable. One suitable particle-mediated transformation is described in PCT published application WO 91/07487. The apparatus used in this technique is commercially available from BioRad.

The following describes the transformation of muscle cells by the method of published PCT application WO 91/00359: Figs. 1 and 2 illustrate an apparatus suitable for particle-mediated transformation of muscle cells and tissues. The present invention makes particular use of the apparatus of Figs. 1 and 2 providing an adjustable electric discharge to physically accelerate DNA coated

onto small particles into the genetic material of muscle cells. The apparatus consists of a spark discharge chamber 12 into which are inserted two electrodes 14 which are spaced apart by a distance of approximately 1 - 2 mm. The spark discharge chamber is a horizontally extended rectangle having two openings 16 and 18 out its upward end. One opening 18 is covered by an access plate 20. The other opening, located opposite from the electrodes 14, is intended to be covered by a carrier sheet 22. The electrodes 14 are connected to a suitable adjustable source of electric discharge voltage. Such a source of electric discharge voltage would preferably include suitable electric switching connected to a capacitor of the 1 to 2 microfarad size range. The amount of the voltage of the charge introduced into the capacitor is adjustable, such as through the use of an autotransformer, through a range of, for example, 1 to 50,000 volts. Suitable switching is provided so that the capacitor can be discharged through the electrodes 14 safely and conveniently by a user.

The carrier sheet 22 is intended to be place upon the opening 18 on the spark discharge chamber 12. Carrier sheet 22 is preferably a sheet of aluminized saran coated mylar. Approximately 5 - 10 millimeters above the opening in the discharge chamber is a retaining screen 24. Placed approximately 5 - 25 millimeters above the retaining screen is a target surface 26.

An exogenous foreign gene construct intended to be transformed into the muscle cells is prepared by suitable DNA or RNA preparation techniques well known to one of ordinary skill in the art. The nucleic acid is dried onto small particles of a durable dense material such as gold, the particles typically being 1 to 3 microns in size. One suitable method of drying the nucleic acid onto the gold particles is described in PCT application WO 91/00359. In brief, the DNA is applied to the particles after precipitation with 25 mM spermidine/6% polyethylene glycol (M.W. 3000) with the addition of $CaCl_2$ to a final concentration of 0.6 M. Preferably, the DNA is coated onto the beads at

a concentration of 0.1-0.5 μg DNA per Mg particles. The coated particles are rinsed in 100% ethanol and applied to the carrier sheet 22.

The coated carrier particles are then placed upon the carrier sheet 22 which is inserted on top of the spark discharge chamber 12. A target tissue, such as muscle cells or tissue, is then placed adjacent to the target surface 26. Then a small droplet of water, approximately 2 - 4 microliters in size, is placed bridging between the ends of the electrodes 14. The access plate cover 20 is then placed over the top of the discharge chamber 12. The atmosphere between the carrier sheet 22 and the target is replaced with helium, by enclosing the apparatus and target and introducing helium in the enclosure in sufficient quantity to largely displace the atmospheric gases.

Appropriate electronic switching may be used to initiate a spark discharge between the electrodes. Preferably, the apparatus is operated at a spark discharge level of 5-15 kV. The force of the electric discharge bridges the spark discharge gap between the electrodes 14 instantly vaporizing the small droplet of water placed there. The force of the vaporization of that water creates a shock wave within the spark discharge chamber 12 which radiates outward in all directions. The impact of the shock wave upon the carrier sheet 22 propels the carrier sheet 22 upwards with great velocity. The traveling carrier sheet 22 accelerates upward in direction until contacting the retaining screen 24. The presence of the helium provides less drag on the flight of the carrier sheet as well as less force for the shock wave to propagate to the target. The carrier sheet 22 is retained at the retaining screen 24, and the DNA-coated particles previously applied to the sheet 22 fly off of the carrier sheet and travel freely on toward the target surface. The particles proceed into the target surface and enter the muscle cells thereon. The momentum of the particles as they impact the surface of the target tissue is adjustable based on the voltage of the initial electric discharge

applied to the electrodes 14. Thus by variations in the amount of electric energy discharged through the electrodes 14, the velocity by which the particles impact the target can be adjusted, and thus the depth of penetration of the particles into the tissue of a target, can be continuously adjusted over the range of adjustment of the electric discharge throughout the electrodes 14.

Implantation and analysis of the implanted transgenic muscle cells are preferably performed as described above. Previous experience with particle-mediated transformation in a variety of cell types has revealed that in most, if not all, cell types a significant level of post-blasting transient gene activity is obtained followed by a decrease in expression to a lower but longer term level of expression. Some of the treated cells become permanently transformed. Thus once the muscle platform cells become capable of long-term survival in the brain, long-term expression of the transgene and delivery to the brain can also be achieved.

CLAIMS

We Claim

1. A method for delivering therapeutic substances to a patient's brain, comprising the steps of:

(a) constructing a nucleotide sequence, wherein said nucleotide sequence is capable of expressing a protein of therapeutic value in the patient's cells;

(b) isolating muscle tissue from the patient;

(c) transforming cells of said muscle tissue with said nucleotide sequence; and

(d) implanting said muscle tissue into the brain of a patient, whereupon the protein is expressed by the nucleotide sequence and a therapeutic substance is produced by the transformed cells.

2. The method of claim 1 wherein the transforming of step (c) is by bombarding the muscle tissue of step (b) with carrier particles coated with the nucleotide sequence of step (a)

3. The method of claim 1 wherein the transforming of step (c) is by liposome-mediated transformation.

4. The method of claim 1 wherein the transformation of step (c) is by injection of a nucleic acid-containing solution into muscle tissue.

5. The method of claim 1 wherein the isolated muscle tissue of step (b) is obtained by mincing muscle.

6. The method of claim 1 wherein the isolated muscle tissue is obtained by enzymatically digesting muscle.

7. The method of claim 1 wherein the implanting of step (d) is into the cortex of the brain.

8. The method of claim 1 wherein the implanting of step (d) is into the caudate nucleus of the brain.

9. The method of claim 1 wherein the patient is a human.

10. The method of claim 1 wherein the isolated muscle tissue is myoblast cells.

11. The method of claim 10 additionally containing the step of culturing the myoblast cells until some of the myoblasts have formed myotubes and wherein the culturing takes place before the transformation of step (c).

12. A method for delivering therapeutic substances to a patient's brain, comprising the steps of:

(a) constructing a nucleotide sequence, wherein said nucleotide sequence is capable of expressing a protein of therapeutic value in the patient's cells;

(b) coating the nucleotide sequence onto carrier particles;

(c) excising muscle tissue from a patient;

(d) accelerating the coated carrier particles into the muscle tissue so as to transform some of the cells in the muscle tissue with the nucleotide sequence, and

(e) implanting the muscle tissue onto the brain of the patient at a location at which the therapeutic substance is desired to be present.

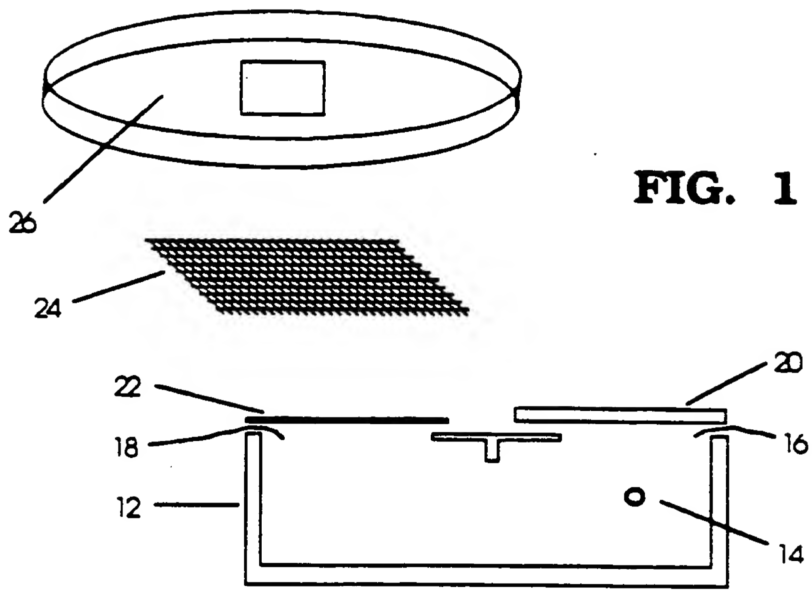

FIG. 1

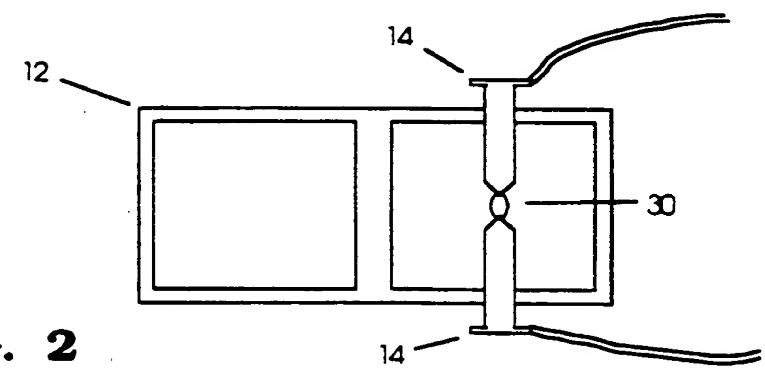

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01815

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :IPC(5): A61K 37/00
US CL :U.S.CL: 424/93

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : U.S.CL: 424/93

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, "transplatation, ""muscle", "brain", author search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Acta Neuropathologica, (Berl), volume 49, no. 3, issued 1980, Heinicke, "Vascular permeability and axonal regeneration in tissues autotransplanted into the brain", pages 175-185, see entire article.	1, 5-8, 10, 11, 12
Y	Molecular and Cellular Biolog, volume 10, no. 6, issued June 1990, Smith <u>et al.</u>, "Genes transferred by retroviral vectors into normal and mutant myoblasts in primary cultures are expressed in myotubes", pages 3268-3271, see entire document.	1, 5-8, 10, 11, 12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 MAY 1993

Date of mailing of the international search report

08 JUN 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer
SUZANNE ZISKA

Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, volume 254, issued 06 December 1991, Barr et al., "Systemic delivery of recombinant proteins by genetically modified fibroblasts", pages 1507-1509, see entire document.	9
Y	WO,A, 91/00359 (Brill et al) 10 January 1991, see entire article.	2, 12
Y	Science, volume 247, issued 23 March 1990, Wolff et al., "Direct gene transfer into mouse muscle in vivo", pages 1465-1468, see entire article.	4
Y	Proceedings of the National Academy of Sciences, volume 84, issued November 1987, Felgner et al., "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure", pages 7413-7417, see entire article.	3